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NEW EXPERIENCE WITH STAPHYLOCOCCAL ENTEROTOXIN

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Food poisonings caused by bacteria or bacterial toxins appear especially in the summertime and sometimes evoke considerable attention, not least in the press. In many cases they are caused by Salmonella-bacteria infected foodstuffs; the etiological background is then easy to establish. Many other organisms such as Cereus bacilli and clostridium can, however, also cause poisonings, but the relationship between the infection and these organisms is not so easy to establish.

Since Dack's [7] investigation in 1930 it has been clear that staphylococci can also cause food poisoning, whereby an enterotoxin manufactured by the staphylococci is believed to be active. Enterotoxin-building strains are found apparently relatively often in human and domestic animal normal bacteria flora [16]. Enterotoxin is perhaps also of importance in staphylococcus enteritis after treatment with antibiotics [6]. Only certain strains manufacture enterotoxin. No easily adapted or good method to prove this, however, as yet exists. In recent years attempts have been made to produce a pure enterotoxin.

Biological Methods

Humans

Of all the mammals it seems that the human is the most sensitive to enterotoxin. A few experiments on man have been performed [9]. The toxin was given orally to nine human subjects; three of these received a severe gastroenteritis, four

received mild symptoms, and two remained healthy. The individual sensitivity thus exhibits individual variations.

Monkeys [27]

Rhesus monkeys (Macaca mulatta) are used. The toxin is given orally with a sound. To compensate for individual variations, six monkeys are used in each experiment; at least two of these must vomit within five hours for the test to be considered positive. Dack believes that the monkey test, although far from satisfactory, is still the best of the biological test methods. This method is similar to the natural way of contracting food poisoning, and the toxin needs no prior preparation. The monkeys show considerable individual variations, and the animals develop relatively quick resistance to the toxin. Sometimes false positive results are obtained.

Cats

Cats as experimental animals were first utilized by Dolman [10] and have since been used in many experiments with good results [15, 21]. Some authors, however, feel that the enterotoxin in cats yields uncertain results [11, 34]. According to the original method the toxin is given intraperitoneally [10], but it can also be given intravenously [15].

In both cases the toxin shall be given about two hours after a meal. Anesthesia is not used, as it can cause nonspecific reactions. With positive results the animal should vomit within two hours. Usually, vomiting occurs within 30 minutes. Regardless of how the test material is given, it must be treated prior to use so that the α-hemolysin is destroyed, as this is highly poisonous to the animal. The prior treatment consists usually of boiling for 30 minutes [8, 29]. With purer enterotoxin preparations the α-hemolysin has been detoxified with formalin or neutralized with the α-antilysin produced with non-toxin producing strains [10, 11, 31]. The disadvantage with the cat test is that the test material must be pre-treated and injected in an unnatural manner. Like other experimental animals, the cat develops resistance to the enterotoxin, and the same cat can be used, at most, three times. The risk of nonspecific reactions is another disadvantage. Nonspecific peritoneal irritation can occur after intraperitoneal injection [14]. The relatively heat-stable β-hemolysin can even give toxin symptoms.

For small laboratories which cannot afford monkeys, it seems that the cat test is the best alternative.

Rabbits

Some authors [22] have with satisfactory results utilized rabbits, which get diarrhea from the enterotoxin. Most researchers [11, 27], however, feel that the rabbit is not satisfactory as an experimental animal. In our own experiments, we have not had any reproducible results.

Frogs

Robinton [23] gave enterotoxin to decerebrated frogs (Rana pipiens) and produced characteristic spasms as a result of antiperistaltic waves. According to others, this phenomena is inconstant and unspecific.

Table 1. Purification of Enterotoxin*

No.	Enterotoxin preparation	Yield/l. bacterial filtrate		Number of antigens by gel-diffusion	Estimated μg. N to cause emesis in 2—4 monkeys in groups of 6
		mg dry wt.	*mg N*		*μg. N/monkey dose*
1	Crude, dialyzed	900	89.1	8	713
2	Crude, pH 3.5 ppt., dialyzed	207	18.9	8	200
3	Crude, pH 3.5 ppt., aluminia adsorption, EtOH ppt., dialyzed	5.7	0.87	7	7.6
4A	Prepn. 3, IRC-50 adsorption, 0.05 M, pH 6.8 eluate; 25 % EtOH ppt.	0.5	0.06	7	1.5
4B	Prepn. 3, IRC-50 adsorption, 0.05 M, pH 6.8 eluate; 30 % EtOH ppt. from 4A supernatant			2	1.1
5A	Prepn. 4A, starch electrophoresis; 30 % EtOH ppt. of toxin zone (zone B, Fig. 2)	0.33	0.042	2	1.2
5B	Prepn. 4A, starch electrophoresis; 30 % EtOH ppt. of fraction at top of toxin peak			1	1.1

* All preparations were lyophilized.

Chick Embryos

Inoculation of enterotoxin to check embryos shows no specific effect [20].

Mice, rats, and guinea pigs are completely resistant [11, 16].

In Vitro Methods

Biochemical Investigations

The enterotoxin is mainly produced by coagulase positive staphylococci [12] but also by coagulase negative ones [33]. All attempts to correlate the toxin to any easily seen characteristic such as gelatin production, salt resistance, β-lysin production, etc., have given negative results [11, 16].

Phage Typing

In meticulous experiments with phage typing most strains reacted with Group III bacteriophages. According to Wallmark [35] most belong to phage type 6/47 or 42D. Similar observations have been made by others. However, this does not mean that all strains with this color pattern produce enterotoxin.

Immunological Methods

Latest research has concentrated on trying to produce an immunological method specific for the enterotoxin. This necessitates a method of producing pure enterotoxin so that it will be possible to produce a specific antitoxin.

Hereby we meet several difficulties. The substrate must have such a composition that good exchange of enterotoxin is obtained, but at the same time it must be completely dialyzable so as not to interfere with later concentratings and purifications. Upon purifying, the separation methods must be such that loss of enterotoxin is minimal and that the biological and immunological characteristics remain unchanged. A few successful results have been reported [1, 2, 4]. However, the existence of enterotoxin directly in poisoned food has never been shown by this method. A tempting method has been described by Sugiyama et al [24]. They cultivate bacteria to be studied on agar into which "pure" anti-enterotoxin has been mixed. After incubation a halo of toxin antitoxin precipitate is formed around the toxin-producing colonies. By noting this halo's size, colonies with strong enterotoxin-producing characteristics can be chosen and cultured in a pure form. Much work remains, however, before an immunological method can be used for routine diagnosis.

Substrate and Culture Methods

Several different media have been used. Dolman et al [10] tested a medium of proteose peptone and 0.3% agar. This medium is believed to be composed to a large extent of complex, non-dialyzable organic compounds which is disadvantageous, especially in immunological investigations. Favorite et al proposed a very simple medium [13]; a casein-hydrolysate with the addition of glucose, nicotinic acid, and thiamine. The toxin exchange, however, was unsatisfactory, so that the medium had to be enriched with meat-peptone boullion upon which complex organic compounds are added, with subsequent disadvantages.

In recent years the substrate of the type recommended by Surgalla et al [30] jointly with Casman [5] have dominated.

Surgalla's medium consists of pancreatin-digested cassein with addition of aneurin and nicotinic acid. Casman used an acid-hydrolized cassein with addition of Ca-pantothenate, aneurin, nicotinic acid, L-cysteine, tryptophane, magnesium sulfate, Na-acetate, and iron.

Toxin production is aided by a 30% CO_2 environment [30] but can be produced even without this [19]. Even stirring the culture aids toxin production [30].

Purification of Enterotoxin

For rough purification several methods have been tested.

The enterotoxin precipitates out with 75--100% saturated ammonium sulfate. Staphylococcal hemolysins precipitate earlier in the lower concentrations [8, 15]. Good results have even been reported with 40% ethyl alcohol at -7°C [3, 8, 32] and with Zn-acetate [3]. When large amounts of culture material were involved (600 liters), it was shown to be most practical to precipitate the enterotoxin with acids, such as H_3PO_4 at a pH of 3.5 [3]. For the final purification one has worked with chromatographic and electrophoretic purification methods. Bergdoll et al, who have probably done the most all-inclusive purification experiments, state in one of their latest works the following modus operandi as the most satisfactory [3, 19].

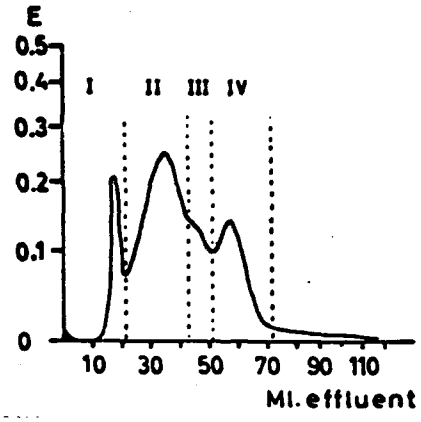

Fig. 1. Purification of concentrated culture supernatant on sephadex G-100. Enterotoxin again found in the second peak (II). Absorption measured at 280 mμ.

Fig. 2. Gel diffusion in modification according to Wadsworth. In the upper pools at left the active sephadex peak (II); the original material, the active cellex-p-peak after precipitation with 0.4 M KPB. In the lower pool commercial Wood serum.

One precipitates first with acid and absorbs on aluminia. The toxin is then eluated with 0.2 M of sodium phosphate buffer. Lately, the first three steps of the procedure have been abandoned because of the loss of toxicity. Now, concentrating of the original supernatant liquid is

performed simply by adsorption on IRC-50, a cation exchange resin, also called Amberlite XE 64. The enterotoxin is eluated with a 0.2 M sodium phosphate solution at pH 6.2. Alcohol precipitation of the eluate at -10°C yields a protein with approximately 20% enterotoxin. Repetition of the procedure yields still more purification up to 70% enterotoxin. It is considered satisfactory if this preparation contains 50% of the original supernatant fluid's enterotoxin activity [2]. For still further purification starch electrophoresis is finally employed. The different fractions were controlled by biological test on a monkey and the purity by the gold diffusion method. After the final purification a product is obtained which gives positive results in monkeys, and only one line in gold diffusion. Not even this fraction is taken to be completely pure; it most likely still contains one or two impurities [3]. One of these is believed to be apyrase [26]. The purest of the active preparations gave positive results in monkeys with a dose of 8--10 mg, which corresponds to one microgram of nitrogen. Before purification the corresponding dose was equal to 713 micrograms of nitrogen (see Table 1). This high-grade purification, however, produced minimal exchange; thus, one usually prefers greater exchange and somewhat less purity. As a comparison, it can be said that Casman [5] was able to produce emesis with as little as 2 micrograms dry weight of a partially purified preparation. Other authors have tried precipitation with 40% ethanol at -20°C followed by iontopheresis on filter paper without any greater success [32].

Enterotoxin Specificity

Of the many extracellular products produced by staphylococci at least the α-lysin and the β-lysin can induce amesis in the cat [14, 29, 31], the α-lysin, however, is quickly destroyed by boiling and apparently cannot then cause emesis. The β-lysin, however, seems to have a certain emetic effect even after boiling and in spite of decreasing hemolysin titres [31]. Some researchers, therefore, wished to assert that the β-lysin and enterotoxin are identical. However, there is no doubt about the uniqueness of enterotoxin. Strains with high β-lysin production do not always produce enterotoxin. Strains without lysin production can produce enterotoxin.

Enterotoxin's Physico-Chemical Characteristics

Through ultracentrifugation and amino acid analysis of purified fractions it has been shown that the toxin is a water-soluble protein with an apparent molecular weight of 23,000 [3].

By electrophoretic studies the isoelectric point has been determined to be at pH 8.5 [3] and with the pH range 4.5 -- 8.2 the toxin is stable for 24 hours at 37° [15]. It resists boiling for 30 minutes but is destroyed to a certain degree after boiling for one hour or autoclaving for 20 minutes [8, 29]. It contains a high lysin content and is trypsin-resistant [3]. In addition, it is stable against alcohol and 0.3% formaldehyde [8, 11] and can stand months of cold.

Immunology

Enterotoxin is a relatively poor antigen. Good rabbit serum has been obtained, however, by intra-muscular injection of antigen combined with Freund's adjuvant intially and then by multiple injections, where the antigen is given intra-peritoneally, intramuscularly, intradermally, subcutaneously, and intravenously, simultaneously [28]. For serological studies some variant of the diffusion method of Oudin or Ouchterlony is usually used. From the investigations performed up to now, it seems that there are different antigenic toxin types.

Sugiyama [24] investigated 29 strains against S6 antitoxin. S6 is a known toxin producer, which Evans isolated for the first time in 1947 in connection with a shrimp poisoning. Of 21 strains which gave positive monkey tests, 10 produced a toxin identical to S6 toxin. None of the eight strains which gave negative monkey tests produced any gel diffusion line with S6 antitoxin. Casman [4] investigated 22 strains, only one of which reacted with S6 antitoxin. This strain was designated type 243. When the strains were tested against 196E antitoxin -- 196E is another known strain which was isolated from ham in 1940 by Slocum -- 13 of them reacted with this antitoxin. S6 toxin also reacted with this antitoxin but not toxin from type 243. As far as can be seen, there are thus now three different antigenic toxins. Neutralizing antibodies against enterotoxin has been shown partly in serum that contains a mixture of antibodies against varied staphylococcus toxins, and partly in specific antienterotoxin serum [5, 28].

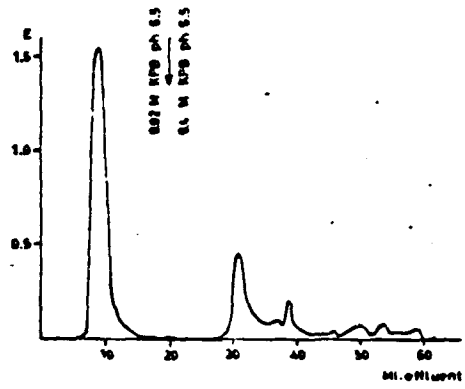

Fig. 3. Continued purification of the second sephadex peak with cellex-p. Enterotoxin obtained with the higher ion concentration. Absorption measured at 280 mμ.

Fig. 4. Gel diffusion in modification according to Wadsworth. In the upper pools from the left, as in Fig. 2. In the lower pools anti-enterotoxin (Bergdoll). As shown in the figure, this anti-serum is not completely "pure." The lower, darker precipitation line should represent the enterotoxin-antitoxin system, while the lighter lines are possibly impurities.

Pharmacological Characteristics

The point of attack for enterotoxin in the human body is still unknown and the way in which enterotoxin works is not known either. After bilateral vagotomy the monkey or cat does not react any longer, which seems to indicate some peripheral, sensory receptor organ. Most likely there also exists a higher center in the fourth ventricle's floor. Bilateral destruction of the posterior area in the fourth ventricle rendered the Rhesus monkey completely toxin-resistant. In view of the fact that animals react so variably, this type of experiment is difficult to evaluate [11, 25].

Private Investigations

At the Bacteriological Institute in Uppsala experiments are being conducted to purify the enterotoxin, among other things, in attempts to find a good laboratory test in vitro. The experiments are still at a preliminary stage, but as we have chosen partly new purification methods, we deemed it of interest to report the experimental setup and some preliminary results.

We used S6- and 196E-strains. Anti-S6-enterotoxin serum was used and purification experiments were made with the S6-strain.

Substrate and Culture Method

Several different media were tested. The best toxin exchange was obtained with a modified Casman's medium [15]. This was composed of ground substrate, but to produce better exchange it was enriched with a dialyzable portion of proteose peptone to a final concentration of 1%, calculated from the dialysate's dry substance. Difcos, a proteose peptone, was used and dialyzed in vacuum by a method described by von Hofsten et al [18]. After dialyzation sterile filtration was performed with a Seitz filter. The culturing itself was done in two steps. In the first step 100 ml graduates were used with sterile glass beads. During incubation in the incubator, the graduates were placed on a tilted, rotating phonograph record. A colony from the blood agar plate or an 18 h boullion culture was used to inoculate the culture. The bacterial culture of the first step was then used as the inoculate in the second step -- the real culturing -- and the inoculate must constitute 1/10 of the final volume. This culturing uses 10-liter graduates and a pulsation culture

technique in a CO_2 medium, according to Heden [17]. On one occasion the culturing was tested on a large scale, 150 liters, at the Karolinska Institute's bacteriological institution with 196E strain. The culturing, which produced good exchange, was done in a CO_2 medium but without pulsation.

Tests in Vitro

Fractions were primarily tested in gel diffusion. We used here Ouchterlony's double diffusion technique in a substance-sparing modification of Wadsworth [34].

Biological Test

On cats intravenous injection according to the technique described by Hammon [15] was tested. The cats had usually been in the institution for a long time before the test in order that they would not give false results. Cat tests were satisfactory and gave reproducible results. The same cat was used for toxin testing at most three times.

Concentrating

The supernatant material from the centrifuged culture was concentrated by vacuum dialysis without any intermediate steps with precipitation. It is possible that this hinders later purification as even other toxins formed by the strain are concentrated at the same time. We have, however, preferred this procedure, in order to lose the least possible toxicity. As original material for the following purification a 500-times concentrated product with a protein content of 1% (calculated according to Lowry) and a α-lysin titre of 1/160,000. That a concentrate of many toxins was present can be ascertained by gel diffusion against Wood-46-Serum (Fig. 2). This is a commercial serum with a large quantity of antibodies against this strain.

Purification

The concentrate which was obtained was further dialyzed against buffers for seven days, after which the product obtained was used as starting material for purification. Two steps were employed here.

1. In a first gel filtration step we used Sephadex G-100 and 0.02 M potassium phosphate buffer, pH 6.8. The fluid which comes out of the sephadex column was gathered in 2 ml fractions. The protein content was continuously measured

in Unicord at 253 mμ. The results of the separation are seen in Fig. 1. Testing in gel diffusion shows enterotoxin again in the other peaks (II). The product is far from pure, which can be seen in Fig. 2, where the original product and the active fractions in both purification steps have been compared in gel diffusion with commercial Wood-serum.

2. Considerably better purification is achieved after the next step, in which a concentrate of the other active sephadex peaks is pushed on the cation exchanger cellex-p in a potassium phosphate buffer with an original concentration of 0.02 M and at a pH of 6.8.

The enterotoxin is "adsorbed" on the column. By then increasing the salt concentration stepwise, we found that the enterotoxin is driven off with 0.06 M to 0.1 M potassium phosphate buffer. In a preliminary experiment this fraction was not given lines against concentrated Wood-serum. Fig. 3 shows an experiment where the enterotoxin has been salted with 0.4 M potassium phosphate buffer. At this high ion concentration, a somewhat lower grade of purification is obtained, which can be seen from Fig. 2. Both the active sephadex- and cellex-p fractions contain enterotoxin, which can be seen from Fig. 4, where the starting material and the active fractions from both steps are compared with a "pure" anti-enterotoxin.

Conclusion

We have shown results where enterotoxin is produced in such pure form that no lines are produced in gel diffusion against commercial Wood-serum. To be considered immunologically pure, however, it must first be tested against antiserum produced through immunization with the original product.

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